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Hydrophobic ion pairing of a minocycline/Ca^{2+}/AOT complex for preparation of drug-loaded PLGA nanoparticles with improved sustained release

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ABSTRACT

Polymeric nanoparticles is an established and efficient means to achieve controlled release of drugs. Incorporation of minocycline, an antibiotic with anti-inflammatory and neuroprotective properties, into biodegradable nanoparticles may therefore provide an efficient means to combat foreign body reactions to implanted electrodes in the brain. However, minocycline is commonly associated with poor encapsulation efficiencies and/or fast release rates due to its high solubility in water. Moreover, minocycline is unstable under conditions of low and high pH, heat and exposure to light, which exacerbate the challenges of encapsulation. In this work drug loaded PLGA nanoparticles were prepared by a modified emulsification-solvent-diffusion technique and characterized for size, drug encapsulation and *in vitro* drug release. A novel hydrophobic ion pair complex of minocycline, Ca^{2+} ions and the anionic surfactant AOT was developed to protect minocycline from degradation and prolong its release. The optimized formulation resulted in particle sizes around 220 nm with an entrapment efficiency of 43% and showed drug release over 30 days in artificial cerebrospinal fluid. The present results constitute a substantial increase in release time compared to what has hitherto been achieved for minocycline and indicate that such particles might provide useful for sustained drug delivery in the CNS.

1. Introduction

Minocycline, a broad-spectrum antibiotic with anti-inflammatory properties, has been shown to have neuroprotective and neurorestorative effects in ischemic injury/stroke and neurodegenerative disease models (Lee et al., 2004; Ryu et al., 2004). The mechanisms behind these effects are believed to lay in minocycline 's ability to inhibit proliferation and activation of reactive microglia (Giuliani et al., 2005; McAllister Ii and Miller, 2010; Tikka et al., 2001). The neuroprotective role of minocycline has been used to improve the quality and longevity of chronic neural recordings by reducing glial sheath growth on implanted microelectrodes in the central nervous system (CNS) (Rennaker et al., 2007). However, the effects were only seen at high doses by conventional administration due to the molecules restricted passage over the blood–brain-barrier (Pardridge, 2005) and short half-life (Di Stefano et al., 2008).

To improve the therapeutic efficacy and decrease the drug dose required from minocycline and other water-soluble drugs, local delivery from biodegradable nanoparticles could be used (Orive et al., 2009). One of the most widely used biodegradable material for drug delivery is poly(D,L-lactic-*co*-glycolic acid) (PLGA) as it undergoes hydrolysis in the body to produce the metabolite monomers, lactic acid and glycolic acid. Since the body effectively deals with these, there is minimal systemic toxicity associated with using PLGA for drug delivery even in the CNS (Houchin and Topp, 2008; Kumari et al., 2010). The PLGA particles can be designed to give a sustained release over a period of days or even weeks after implantation. Hence, such a sustained release of minocycline from nanoparticles would be ideal for reducing the foreign body response seen after implanting electrodes in the brain (Polikov et al., 2005).

Encapsulation of water-soluble drugs into PLGA nanoparticles is generally done by double emulsion techniques (Astete and Sabliov, 2006). This approach often yields high encapsulation efficiencies

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but has the drawback of creating porous particles that are rapidly wetted upon contact with aqueous media, which subsequently results in faster release rates (Fredenberg et al., 2011; Siepmann et al., 2005). Using homogenous particles where the drug is entrapped throughout the particle matrix is preferable in this sense (Klose et al., 2006; Stevanović and Uskoković, 2009). Preparation of these is usually based on single oil-in-water (o/w) emulsification methods, such as the emulsification-solvent-diffusion (ESD) technique (Kwon et al., 2001; Pinto Reis et al., 2006; Quintanar-Guerrero et al., 1997) schematically shown in Fig. 1. Both drug and polymer are first dissolved in an organic solvent followed by emulsification into an aqueous phase containing a surfactant. A large amount of water is then added to the system leading to solvent diffusion to the external phase and the formation of nanospheres. Finally the solvent is evaporated, resulting in a stable colloidal suspension containing nanoparticles of solid polymer stabilized by the surfactant. The surfactant type and concentration will have a large impact on the final particle size and affects the drug loading efficiency (Gómez-Gaete et al., 2007), sphericity (Esposito et al., 1999) and surface charge of the formed particles (Italia et al., 2007). A commonly used surfactant for this preparation method is didodecyldimethylammonium bromide (DMAB), a double-chain compound belonging to the class of cationic quaternary ammonium surfactants (Kwon et al., 2001). Cationic surface charges are desirable as it promotes interaction of the nanoparticles with cells and hence increases the rate and extent of internalization (Kumari et al., 2010). The ESD technique presents several advantages, such as high batch-to-batch reproducibility, simplicity and narrow size distribution. Disadvantages are the large volumes of water to be eliminated from the suspension and the leakage of water-soluble drugs into the aqueous external phase during emulsification. Drug leakage will result in low encapsulation and can lead to a high initial burst release (Danhier et al., 2012) if the leaked drug adsorbs onto the surface of the particles rather than being dispersed throughout the polymer matrix. Another contributing factor for low encapsulation efficiency is degradation of the drug during preparation (Xu et al., 2009). Minocycline is known to be unstable under acidic or alkaline conditions, heat and exposure to light, where it undergoes epimerization and degrades (Chow et al., 2008; Zbinovsky and Chrekian, 1977). Studies have however shown that chelation of minocycline with Ca^{2+} significantly enhances the stability of the drug in solution (Soliman et al., 2010).

Unlike other tetracyclines, minocycline contains two amino groups that are responsible for its high solubility in water. In total, it has four ionizable groups with the dissociation constants of $pK_{a1} = 2.8$; $pK_{a2} = 5.0$; $pK_{a3} = 7.8$; and $pK_{a4} = 9.3$ (Kalish and Koujak, 2004). The isoelectric point of minocycline is at pH 6.4 were the molecules neutral zwitterionic form is predominant and the highest partition coefficient in octanol is found (Zbinovsky and Chrekian, 1977). The high aqueous solubility of ionic compounds stems not only from being charged, but also from the fact that the counter ions easily solvates by water. If the counter ion would be replaced with a species with similar charge, but less solvated by water it is expected that the aqueous solubility would decrease. This process is referred to as hydrophobic ion pairing (HIP) (Jacobson et al., 2010; Meyer and Manning, 1998) and involves stoichiometric replacement of polar counter ions with ionic detergents of similar charge.

The purpose of this study was to prepare DMAB stabilized minocycline-loaded PLGA nanoparticles by the ESD-method, aiming for particle sizes around 100 nm, high encapsulation efficiencies and long release times. We have evaluated two approaches to increase minocycline's solubility in organic solvents by replacing the water-soluble hydrochloride salt form of minocycline with either its isoelectric form at pH 6.4 or a HIP-complex of minocycline, Ca^{2+} and the anionic surfactant bis(2-ethylhexyl) sulfosuccinate (AOT), an FDA approved food additive (Fig. 1C). The prepared nanoparticles were characterized for size, morphology, drug loading and *in vitro* drug release.

2. Materials and methods

All samples were prepared in triplicate and tested with triple measurements or more. Data is presented as the mean ± standard deviation of the mean.

2.1. Materials

Poly(D,L-lactic-co-glycolic acid) (PLGA) (50:50), Mw 24000-38000 acid terminated (Sigma). Minocycline hydrochloride (Sigma–Aldrich). Didodecyldimethylammonium bromide (DMAB)

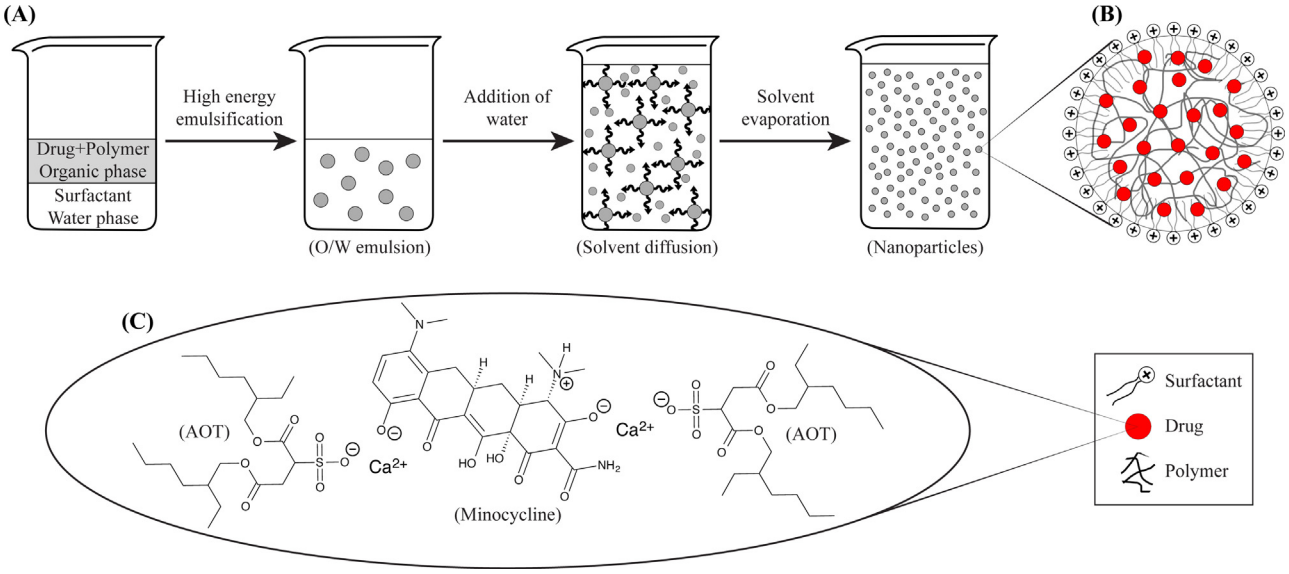

Fig. 1. (A) Schematic representation of PLGA nanoparticle preparation by the ESD-technique. (B) Proposed composition of the formed drug-loaded nanoparticles; the entrapped drug is distributed throughout the polymer matrix and the particles surface is covered with the cationic surfactant DMAB. (C) HIP-complex of minocycline, Ca^{2+} ions and bis(2-ethylhexyl) sulfosuccinate (AOT).

(Sigma–Aldrich) was used as stabilizer. Sodium bis(2-ethylhexyl) sulfosuccinate (AOT) (D4422, Sigma–Aldrich) was used as an ion pairing agent. HEPES-buffered artificial cerebrospinal fluid pH 7.3 (aCSF) (Protocols, 2011) was used as drug release medium. All other chemicals and solvents used were of HPLC grade. Milli Q ultra-pure water (Millipore) was exclusively used for the preparation of all aqueous solutions.

2.2. Preparation of nanoparticles

Nanoparticles were prepared by an ESD technique (Quintanar-Guerrero et al., 1997). In brief, PLGA (20 mg) was dissolved in an ethyl acetate/methanol mixture (9:1, 1 ml) at room temperature. The organic phase was added drop-wise to a bis–tris buffered aqueous solution (10 mM, pH 6.4) containing a stabilizer (DMAB, 0.10%, 1.2 ml) under magnetic stirring at 600 rmp. The two-phase system was then emulsified by sonication for 10 minutes using an iced ultra-sonic cleaning bath (VWR USC 300 D, 80 W at 45 kHz). To this emulsion, water (12 ml) was added under constant stirring, which resulted in nanoprecipitation. The suspension was then kept in an open beaker with magnetic stirring (400–500 rpm) overnight to evaporate the organic solvent. To prepare drug-loaded nanoparticles, minocycline was dissolved together with PLGA in the organic phase following the same protocol. The influence of altering the following formulation parameters was investigated and optimized with respect to particle size and drug incorporation, aiming for particle sizes around 100 nm and high encapsulation efficiency.

DMAB concentration

Blank nanoparticles were prepared using different DMAB concentrations (0.01–0.15%) in bis–tris buffered water phase (10 mM, pH 6.4) and then evaluated for size and zeta potential.

Preparation of nanoparticles with the isoelectric form of minocycline at pH 6.4

Minocycline was dissolved in water and set to its isoelectric point at pH 6.4 by addition of 0.2 M NaOH. Minocycline at its isoelectric point was extracted with dichloromethane (DCM) and the solvent was evaporated under reduced pressure. Particle preparation was performed as described above and three different drug-to-polymer (D/P) ratios (5, 12.5 and 20%) were evaluated.

Preparation of nanoparticles with a HIP complex of minocycline, Ca^{2+} and AOT

The HIP complexation was accomplished according to Soliman et al. (2010) and the Bligh-Dyer method (Bligh and Dyer, 1959) in a monophase of dichloromethane, water, and methanol. Stock solutions of $CaCl_2$ and minocycline were prepared in Tris–HCl buffer (10^{-2} M, pH 7.4). The pH of the solutions was adjusted to 7.4 using 0.1 M NaOH, if necessary. The $CaCl_2$ solution was added to the minocycline solution at specific volume to attain a minocycline/Ca^{2+} molar ratio of 1:2. This solution was then added to an equal volume of DCM containing an equimolar amount of AOT with respect to the Ca^{2+} ions. A mono-phase was formed by addition of methanol at volume ratios of 1:1:2.1 of (buffer/DCM/methanol). The solution mixture was finally phase separated by addition of equal volumes of buffer (1 ml) and DCM (1 ml) followed by centrifugation at 1000 rcf for 1.5 minutes, with the minocycline/Ca^{2+}/AOT-complex now present in the organic phase. The complex was isolated by separation of the two phases and evaporation of the solvent. ^{1}H NMR spectra of the formed complex were recorded on a Bruker Avance II 400 MHz spectrometer in deuterated chloroform ($CDCl_3$) at room temperature. Chemical shifts are given in ppm downfield from the signal for Me_4Si, with reference to residual $CDCl_3$. Control samples of minocycline, minocycline/Ca^{2+} (molar ratio 1:2), AOT, and minocycline/AOT (molar ratio 1:2) all subjected to the same method were also recorded. Particle preparation was performed as described above except for the use of Tris–HCl buffer (10^{-2} M, pH 7.4) instead of bis–tris buffer in all aqueous solutions. Five different *D*/*P* ratios (1, 1.5, 2, 3 and 4%) were evaluated.

2.3. Particle size and morphology

The hydrodynamic size, polydispersity index (PDI) and zeta potential of the prepared nanoparticles was determined by dynamic light scattering (Malvern Zetasizer Nano-ZS), taking the average of three measurements for each batch. The suspensions were diluted ten times with the aqueous phase used in the formulation before measurements. The morphology of the nanoparticles was investigated by SEM (FEI Nova NanoLab 600) at a working distance to 5.2 mm and an accelerating voltage of 15 kV. The sample was prepared by spin-coating the filtered crude suspension on a silicon wafer at 2000 rpm for 1 min.

2.4. Drug encapsulation

Centrifugation was used to separate the drug-loaded nanoparticles from the residual free drug in the suspensions. All nanoparticle suspensions were filtered through a sintered glass filter before the separation. The suspensions were centrifuged (Centrifuge 5430, Eppendorf) at 7197 rcf for 30 min, the supernatant was discarded and the pellet was resuspended in pure water, three times. The washed nanoparticles were finally frozen in liquid nitrogen and lyophilized at −55 °C and 0.050 mbar for 12 h (Freezone 4.5 model 77510, Labconco). The freeze-dried nanoparticles (NPs) were weighted and dissolved in acetonitrile (0.1% TFA, 400 μl). Methanol (0.1% TFA, 1 ml) was then added to dissolve the drug and precipitate the polymer. The solution was centrifuged at 1000 rcf for 5 min and the supernatant was filtered through a 0.45 μm Teflon filter. The amount of minocycline was quantified by HPLC (Hewlett-Packard LC 1100 Series, Agilent Technologies, Foster City, CA, USA) at 25 °C using a Xterra MS 50 × 2.1 mm, 2.5 μm particle size reversed phase C18 column (Waters, Millford, CT, USA). A mobile phase gradient program was used starting at 95% trifluoroacetic acid (0.05%), gradually changing over to 30% acetonitrile in 4 min and then finishing by changing to 95% acetonitrile in one minute at the flow rate of 0.27 ml/min. An injection volume of 5 μl and a detection wavelength of 354 nm were used. Linear calibration curves ($r^2 \geq 0.999$) were obtained over the drug concentration range of 2–20 μg/ml.

The drug encapsulation is expressed both as drug content (DC) and entrapment efficiency (EE), represented by Eqs. (1) and (2) and respectively.

$$\text{DC}\,(\%) = \frac{\text{mass of drug in NPs}}{\text{mass of freeze} - \text{dried NPs}} \times 100 \quad (1)$$

$$\text{EE}\,(\%) = \frac{\text{actual drug content}}{\text{theoretical drug content}} \times 100 \quad (2)$$

2.5. Drug release

The drug release from the optimized 3% minocycline/Ca^{2+}/AOT-formulation was studied *in vitro* using a dialysis technique. Three batches of freshly prepared nanoparticle suspensions (total volume of 42 ml, containing 60 mg PLGA and 1.8 mg minocycline) were combined, filtered through a glass filter and lyophilized. Mannitol (2% w/v) and Pluronic F-127 (1%, 3 ml) were used as cryoprotectants to enable redispersion and prevent particle aggregation. The freeze-dried nanoparticles were resuspended in 5 ml of aCSF and placed in a dialysis bag (SnakeSkin, 10K MWCO, Thermo Scientific, Rockford, IL, USA). The dialysis bag was then

immersed in a amber beaker containing 35 ml aCSF and maintained at 37 °C in a water bath. At predetermined time intervals, the whole release medium was replaced with fresh medium. The released amount of minocycline was quantified by HPLC at 25 °C using a Sunfire 100 × 3.0 mm, 3.5 μm particle size reversed phase C18 column (Waters, Millford, CT, USA). An isocratic mobile phase of trifluoroacetic acid (0.05%)-acetonitrile at the volume ratio of 87:13 and flow rate of 0.6 ml/min was used. All samples were filtered through a 0.20 μm Teflon filter. An injection volume of 20 μl and a detection wavelength of 354 nm were used. Linear calibration curves ($r^2 \geq 0.980$) were obtained over the drug concentration range of 1–10 μg/ml. Evaluation of the dialysis method described above was done by measuring minocycline's diffusion over the dialysis bags in two different setups; minocycline only and minocycline together with cryoprotectants in aCSF. The stability and degradation of minocycline in aCSF (25, 50 and 100 μg/ml) under the *in vitro* release conditions was also investigated in a seven-day study.

3. Results and discussion

3.1. Particle size and morphology

Initial studies were made on the surfactants effect on size and morphology. Drug free nanoparticles were prepared using different DMAB concentrations and evaluated for size, PDI and zeta potential. The PDI is a dimensionless measure of the broadness of the size distribution calculated from a cumulants analysis of the DLS-measured intensity autocorrelation function. In the Zetasizer software the PDI ranges from 0 to 1. Numerically, a dispersion may be considered monodisperse if 90% of the distribution lies within ±5% of the average size. The PDI typically has values less than 0.1 for a monodisperse test sample (ISO-22412:2008). Values greater than 0.7 indicate that the sample has a very broad size distribution and is probably not suitable for the dynamic light scattering (DLS) technique. As can be seen in Table 1, formulations with DMAB concentrations lower than 0.10% resulted in larger particles with broader size distributions (PDI > 0.1), negative zeta potentials and/or unsuccessful emulsions. Formulations with DMAB concentrations of 0.10% and higher resulted in monodisperse nanoparticles (PDI < 0.1) with positive zeta potentials. The mean particle size was 150 nm in diameter and decreasing with increasing surfactant concentrations.

The amount of surfactant used during particle preparation is an important factor for both size reduction and stability of the prepared nanoparticle suspensions. Too low concentration leads to aggregation of the polymer, whereas too high concentration may affect drug incorporation as a result of the interaction between drug and stabilizer. The zeta potential gives information on the stability of the prepared nanoparticle suspensions and is strongly influenced by the particles surface charge and hence the type of surfactant being used. Particles with large negative or positive zeta potentials (>± 30 mV) will form stable colloidal suspensions since they tend to repel each other and thereby reduce aggregation.

Table 1
Effect of DMAB concentration on size, PDI and zeta potential (mean ± s.d., $n = 3$).

DMAB conc. (% w/v)	Size (d. nm)	PDI	Zeta pot. (mV)
0.01	Aggregation	–	–
0.025	Aggregation	–	–
0.05	900 ± 200	0.50 ± 0.08	−58 ± 7
0.075	3400 ± 500	0.39 ± 0.09	−2 ± 8
0.10	150 ± 10	0.06 ± 0.02	+57 ± 7
0.125	140 ± 2	0.05 ± 0.02	+75 ± 4
0.15	124 ± 2	0.06 ± 0.01	+80 ± 5

Generally, PLGA nanoparticles generate negative zeta potentials due to the PLGA's free carboxylic end groups (Kuo and Yu, 2011; Stolnik et al., 1995). Here, the zeta potential shifted to positive values with increasing surfactant concentration most likely as an effect of the increasing surface coverage of DMAB on the prepared particles.

It should be noted that the 0.05% and 0.075% formulations initially formed visibly stable suspensions. These suspensions, however, started to flocculate with time and the reported sizes are more likely of agglomerated nanoparticles, as indicated by their higher PDI-values. A possible explanation is that the amount of surfactant was sufficient to stabilize the dispersed droplets of solvent in the o/w emulsion and enable the nanoparticle formation during preparation but insufficient to stabilize the formed nanoparticles in the final suspension. Therefore the zeta potential for these formulations more likely represents that of the formed agglomerates and not of the nanoparticles.

The surfactant concentration should be kept to a minimum to reduce the risk of interactions between drug and surfactant, but yet high enough to prevent particle aggregation stabilize the suspensions. A DMAB concentration of 0.10% was therefore used in all subsequent formulations. Our optimized HIP-complex formulation with a theoretical D/P ratio of 3% generated a positive zeta potential of 55 ± 4 mV and somewhat larger particles as compared to the blank formulation (220 nm and 150 nm, respectively). This is illustrated by the size distribution curves generated via DLS in Fig. 2. The more or less unchanged zeta potential of the HIP-complex formulation compared to the blank (+55 mV and +57 mV, respectively), indicate that there was no interaction between drug and particle surface.

All size measurements were performed using DLS, which reports the size of a hypothetical hard sphere that diffuses in the same fashion as that of the particle being measured. In order to validate the size results obtained with DLS, the overall shape of the nanoparticles was investigated by SEM imaging. The SEM micrographs in Fig. 2 confirm that both the blank and the HIP-complex loaded nanoparticles were spherical.

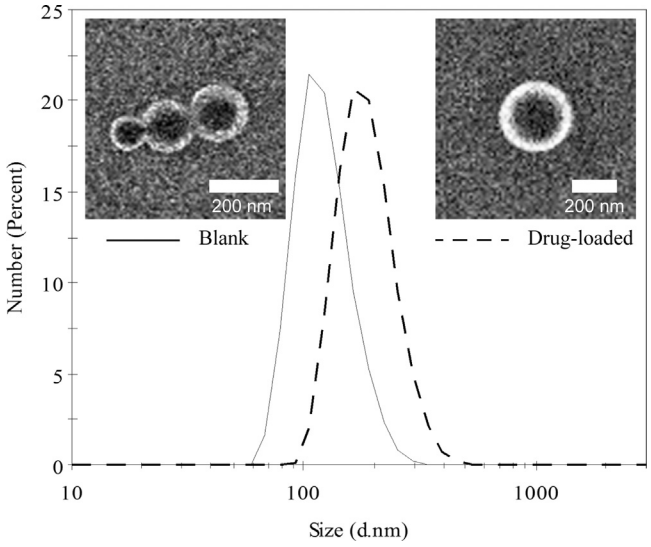

Fig. 2. DLS size distribution curves and SEM micrographs of blank and drug-loaded* PLGA nanoparticles prepared with the same DMAB concentration (0.10%). Note that the number of image pixels and signal-to-noise ratio are limited for these SEM images due to a high sensitivity of the nanoparticles to radiation damage. *A minocycline/Ca^{2+}/AOT-complex at a theoretical D/P ratio of 3% was used.

Table 2
Effect of drug form and *D*/*P* ratio on size, PDI, drug content (DC) and entrapment efficiency (EE) (mean ± s.d. $n = 3$).

Drug form	*D*/*P* ratio (%)	Size (d. nm)	PDI	DC (%)	EE (%)
MC pH 6.4	5	159 ± 4	0.04 ± 0.01	0.06 ± 0.01	1.4 ± 0.2
	12.5	161 ± 2	0.04 ± 0.02	0.16 ± 0.02	1.5 ± 0.2
	20	145 ± 4	0.05 ± 0.02	0.26 ± 0.03	1.6 ± 0.2
MC/Ca^{2+}/AOT	1	180 ± 5	0.04 ± 0.02	0.11 ± 0.02	12 ± 2
	1.5	190 ± 10	0.08 ± 0.03	0.27 ± 0.05	20 ± 4
	2	195 ± 1	0.06 ± 0.01	0.82 ± 0.04	43 ± 2
	3	220 ± 6	0.07 ± 0.04	1.12 ± 0.01	43 ± 1
	4	700 ± 600	0.4 ± 0.1	–	–

3.2. Drug encapsulation

To obtain nanoparticles with high entrapment efficiencies by the ESD-method, a key parameter is controlling the drugs solubility in organic solvents. To increase minocycline's (MC) lipophilicity, we attempted to replace the hydrochloride salt form with either the isoelectric form (MC pH 6.4) or the HIP-complex (MC/Ca^{2+}/AOT). Furthermore, different D/P ratios were evaluated for optimizing the drug encapsulation. Table 2 summarizes the effect of different combinations on particle size, PDI, drug content and entrapment efficiency. All formulations except the 4% HIP-complex, resulted in monodisperse particles (PDI < 0.1) with mean sizes below 220 nm. The lowest entrapment efficiencies were obtained by the isoelectric approach, most likely because the drug is still very water-soluble in the zwitterionic state and diffuses easily into the external aqueous phase during preparation. The isoelectric formulations were therefore excluded from all further characterization. The highest drug content and entrapment efficiency (1.12% and 43%, respectively) were achieved with the HIP-complex in combination with a D/P ratio of 3%. This compares with others in the field who have achieved 1.92% and 29.95%, respectively, for ion paired PLGA–PEG-dextran sulphate nanoparticles (Kashi et al., 2012) and 9.3% and 46.5%, respectively, for sodium cholate stabilized PEG–PLA nanoparticles (Yao et al., 2014). ^{1}H NMR spectra of the HIP-complex and control samples are shown in Fig. 3. Comparison of chromatogram (A) and (B) show changes in intensity and shape for minocycline's characteristic peaks at 5.95, 6.83, 7.31, 9.50, 11.53 and 14.87 ppm, which indicates minocycline complexation with Ca^{2+} ions. Comparison of chromatogram (C) and (D) show a broadening of AOT's characteristic peaks at 3.74–3.98 ppm in the presence of the minocycline/Ca^{2+} chelate, strongly suggesting that a minocycline/Ca^{2+}/AOT-complex has formed. Chromatogram (E) of AOT and minocycline in the absences of Ca^{2+} appears as a combination of chromatogram (A) and (C), indicating no interaction occurs between minocycline and AOT in the absence of Ca^{2+} ions.

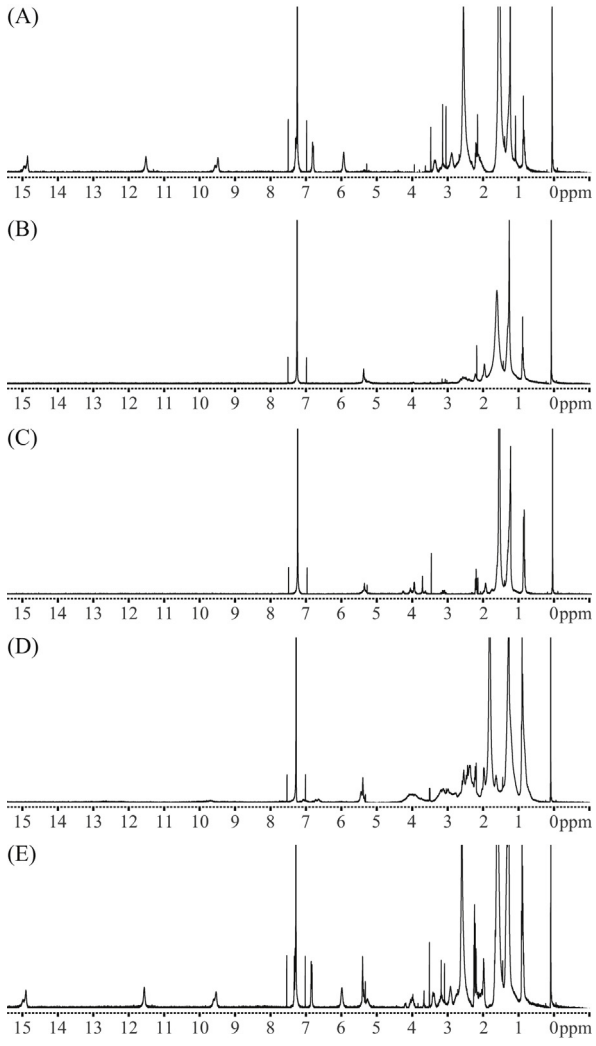

Fig. 3. ^{1}H NMR spectra in $CDCl_3$ at room temperature: (A) MC; (B) MC/Ca^{2+}; (C) AOT; (D) MC/Ca^{2+}/AOT; (E) MC/AOT.

Two different buffering agents were used during particle preparation, as they are useful in different pH ranges; bis–tris (pH range 5.8–7.2) was used in the blank and iso-electric formulations at pH 6.4, and Tris–HCl (pH range 7–9) was used in the HIP-complex formulations at pH 7.4. The choice of buffering agent could have effects on particle characteristics such as size and drug content. The results presented in Tables 1 and 2 however, indicate that the size increase seen for HIP-complex loaded nanoparticles is more likely an effect of increased drug content rather than an effect of using a different buffering agent.

3.3. In vitro *drug release*

The drug release was quantified *in vitro* by a dialysis method. Unwashed nanoparticles were used both to mimic the future handling of the formulations, and to prevent morphological changes and premature drug release, which a washing step using centrifugal forces might induce (Moreno-Bautista and Tam, 2011). The release profile of minocycline from minocycline/Ca^{2+}/AOT-complex loaded PLGA nanoparticles with a theoretical D/P ratio of 3% (Fig. 4) show an initial burst during the first 24 h followed by a linear release over 30 days. Since unwashed particles were used, the initial spike in drug release is due to unencapsulated drug. The subsequent slow release is more likely caused by drug diffusion and erosion of the polymeric matrix. Cryoprotectants were added before freeze-drying to prevent the nanoparticles from agglomerating and should be regarded as a part of the formulation. After resuspension, the initial mannitol and Pluronic F-127 concentration inside the dialysis bags were approx. 22% and 0.5%, respectively. Effect of cryoprotectants on minocycline's diffusion through the dialysis bags and the release curves are shown in Fig. 4. The release for only minocycline show that more than 98% of the free drug was released within two days, which follows the expected release kinetics from a dialysis bag when replacing the surrounding release medium at each sampling point and assuming unhindered diffusion. When cryoprotectants were present however, six days were required for 98% of total drug content release, which most likely is an effect of the initial high mannitol

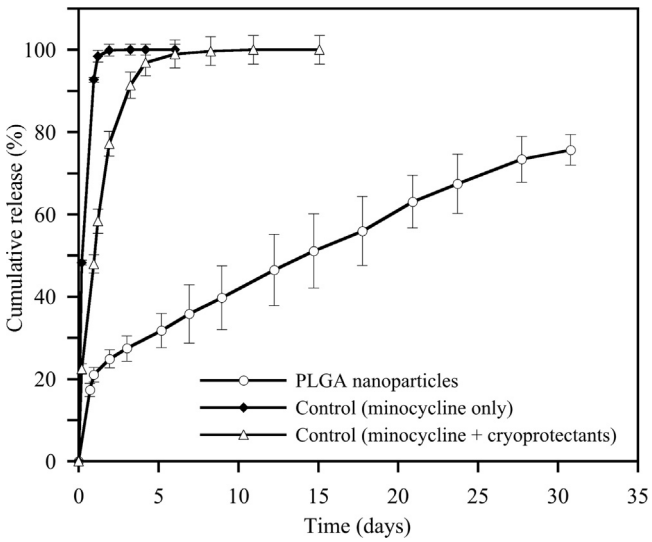

Fig. 4. *In vitro* release from minocycline/Ca^{2+}/AOT-complex loaded PLGA nanoparticles with a theoretical D/P ratio of 3% and control curves of minocycline only and minocycline together with cryoprotectants.

concentration inside the dialysis bags that causes an increased viscosity and thereby a slower intrinsic diffusion. Pluronic F-127 is not likely to affect the viscosity at such low concentrations (Gilbert et al., 1986). The slower intrinsic diffusion is also likely to occur in the nanoparticle release study but will presumably level of with time as the mannitol molecule is small enough to diffuse through the dialysis tubing. There were no measured differences in replaced amount of release medium between the three different cases and no visual observations of osmotic pressure building up inside the bags during the release study, which also supports this assumption. The minocycline concentration was evidently higher inside than outside the dialysis bag during the first sampling points when cryoprotecants were present. This could slow down the diffusion of drug molecule from the drug carrier as the concentration gradient between inside particle and the interior of dialysis bag is lower. Due to these diffusional barriers the initial drug release may be lower in the generated curve than it would be if samples ware taken from inside the dialysis bags. This may in turn also slow down the overall release from the particles and give rise to a slightly longer release profile. The choice of aCSF as release medium stemmed not only from the prospective application area, the brain, but also for its high content of protective divalent cations (Mg^{2+} and Ca^{2+}). No degradation of minocycline was detected in aCSF during the seven-day stability study (data not shown). Furthermore, since the sampling interval was never longer than three days and the entire release medium outside the dialysis bags was replaced at each sampling time, there was no need to compensate the release curve for degradation. The fact that minocycline was detectable even after 30 days suggests that the HIP-complex was dispersed throughout the polymeric matrix rather than adsorbed to the surface of the particles. The long release time also suggest that minocycline is protected from degradation when encapsulated in this form, despite the risk of creating an acidic milieu inside the particle as the polymer is hydrolysed (Houchin and Topp, 2008).

The present results constitute a substantial increase in release time compared to what has hitherto been achieved for minocycline-loaded polymeric nanoparticles. For example, Kashi et al. (2012), who investigated different methods for preparing PLGA nanoparticles containing minocycline, showed an *in vitro* drug release in phosphate- buffered saline (PBS; pH 7.4) over five days by an ion pairing technique with PLGA-PEG-dextran sulphate. Yao et al. (2014) reported a release time of 14 days in PBS (0.01 M, pH 7.4) from their sodium cholate stabilized PEG-PLA nanoparticles which could be ideal for periodontal disease treatment. Our longer release profile over 30 days, however, provide a better match with respect to the normal time course of brain tissue responses associated with implantation of electrodes (Polikov et al., 2005). It should be noted that a comparison of *in vitro* drug-release profiles with others could be misleading, as no standard testing procedures are used. The *in vitro* release methods and test conditions are often chosen with regard to the prospective environment the colloids are intended for, which may affect the release kinetics. The biological activity of the released minocycline from our minocycline/Ca^{2+}/AOT loaded PLGA nanoparticles remains to be evaluated *in vivo*. Prior to clinical use, aspects such as surfactant toxicity and residual solvent content should also be assessed (Dixit et al., 2015).

4. Conclusion

In this work, minocycline-loaded PLGA nanoparticles were prepared by a single oil-in-water based preparation technique and characterized for size, drug entrapment and *in vitro* drug release. The influence of various formulation parameters was investigated and optimized with respect to particle size and drug incorporation. Introduction of a novel HIP-complex of minocycline, Ca^{2+} and AOT resulted in monodisperse particles around 220 nm in size, high encapsulation efficiency (43%) and long *in vitro* drug release (>30 days). Our results demonstrate that hydrophobic ion pairing with AOT is an effective strategy to incorporate minocycline into PLGA nanoparticles and could further be applicable to other water-soluble drugs. The high encapsulation efficiency and prolonged release profile in artificial CSF suggests that the particles may be useful to accomplish sustained and local delivery of minocycline in the CNS. We propose that such nanoparticles delivered from implanted electrodes in the brain may be used to reduce the foreign body responses and protect the nearby neurones.

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